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Author contact	your email lizanne.janssens@bio.kuleuven.be your phone number *+ 32 (0)16 32 38 57*
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14 Reinforcing effects of non-pathogenic bacteria and predation risk: from physiology to life
15 history
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17 Lizanne Janssens[1*], Robby Stoks[1]
18 [1] Laboratory of Aquatic Ecology, Evolution and Conservation, University of Leuven, Charles
19 Deberiotstraat 32, B-3000 Leuven, Belgium
20
21 * Corresponding author: Lizanne Janssens
22 Tel. +3216323857; fax: +3216324575
23 E-mail address: lizanne.janssens@bio.kuleuven.be
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Abstract

The important ecological role of predation risk in shaping populations, communities and ecosystems is becoming increasingly clear. In this context, synergistic effects between predation risk and other natural stressors on prey organisms are gaining attention. Although non-pathogenic bacteria can be widespread in aquatic ecosystems their role in mediating effects of predation risk has been ignored. We here address the hypothesis that non-pathogenic bacteria may reinforce the negative effects of predation risk in larvae of the damselfly *Coenagrion puella*. We found synergistic effects for all three life history variables studied: mortality increased, growth reductions were magnified and bacterial load was higher when both non-lethal stressors were combined. The combined exposure to the bacterium and predation risk considerably impaired the two key antipredator mechanisms of the damselfly larvae: they no longer reduced their food intake under predation risk and showed a synergistic reduction in escape swimming speed. The reinforcing negative effects on the fitness-related traits could be explained by the observed synergistic effects on food intake, swimming muscle mass, immune function and oxidative damage. These are likely widespread consequences of energetic constraints and increased metabolic rates associated with the fight-or-flight response. We therefore hypothesize that the here documented synergistic interactions with non-pathogenic bacteria may be widespread. Our results highlight the ignored ecological role of non-pathogenic bacteria in reinforcing the negative effects of predation risk on prey organisms.

Key words: damselfly larvae, non-consumptive effects, oxidative damage, predator-prey interactions, synergistic effects

Introduction

Ecologists are increasingly aware that besides consumptive effects, predators may also impose non-consumptive effects on prey (Preisser et al. 2005) and thereby acknowledge the important ecological role of predation risk in shaping prey population dynamics (McCauley et al 2011), community structure (Peacor et al. 2012) and ecosystem functions (Hawlena et al. 2010). In this context, synergistic effects between predation risk and other natural enemies on prey organisms are gaining attention (Decaestecker et al. 2002; Yin et al. 2011). Because of the general belief that widespread non-pathogenic bacteria do not impair fitness-related traits, their potential to modulate predator effects has been ignored. Yet, recently it has been documented that non-pathogenic bacteria (Freitak et al. 2007, 2009) as well as the non-pathogenic cell wall compounds of gram-negative bacteria (Ahmed et al. 2002; Kelly 2011) can negatively affect fitness-related traits.

There are different mechanisms through which non-pathogenic aquatic bacteria can cause stress to aquatic animals: (i) they may impose energetic constraints associated with the upregulation of the immune defence (Freitak et al. 2007, 2009). (ii) Bacteria can cause increased Reactive Oxygen Species (ROS) concentrations due to activation of the immune system (Steinberg 2012) or by ROS production by the bacteria themselves (Shaked and Rose 2013). High levels of oxidative damage can impair critical functions such as signal transduction and the functioning of receptors, antibodies and transport proteins and enzymes (Halliwell and Gutteridge 2007). (iii) Competition for resources between the natural symbiotic midgut microbiota and the bacteria admitted to the animals (Smith and Holt 1996), may reduce fitness-related traits such as growth rate. (iv) Bacterial growth in the water phase and the associated oxygen uptake en nitrogen excretion may change abiotic conditions and thereby also cause stress to the animals (Ansa et al. 2011).

Since predation risk also can cause energetic constraints (Stoks et al. 2005b) and oxidative damage (Janssens and Stoks 2013a), this generates the question whether non-pathogenic bacteria may also reinforce the effects of predation risk. This could have important consequences for ecosystem dynamics in aquatic systems where predators (Kerfoot and Sih 1987) and non-pathogenic bacteria (Leff and Lemke 1998) are omnipresent. Interactions between stressors have mostly been studied at the level of life history traits with a strong bias towards effects on mortality, while effects on performance and physiology are often neglected (Darling and Côté 2008). The latter types of effects can also have important ecological consequences. Negative effects on a prey's escape swimming performance will increase vulnerability to predation (e.g. Stoks and McPeek 2003; Strobbe et al. 2009, 2010; Janssens and Stoks 2012) and thereby influence the predator-prey interactions in the ecosystem. Stressors that cause physiological changes leading to oxidative damage may have profound fitness consequences as they can reduce reproductive output and accelerate ageing (Monaghan et al. 2009). Although both predation risk (Guerra et al. 2013; Janssens and Stoks 2013a) and pathogenic bacteria (Baik et al. 1996; Castex et al. 2009; Wang et al. 2012) have been shown to cause oxidative damage to biomolecules, so far no studies looked at the impact of non-pathogenic bacteria and their combined effect with predation risk on oxidative damage.

In this study we will investigate the hypothesis that predation risk and exposure to non-pathogenic bacteria interact in affecting fitness-related traits of aquatic prey organisms. As the presence of interactions between stressors may depend on the type of response variable (Janssens & Stoks 2013b), and to get a multifaceted view of the combined impact of predation risk and exposure to non-pathogenic bacteria we will study effects at different trait types. We will look at effects on (i) life history (survival, growth rate, internal bacterial load), (ii) behaviour (food intake), (iii) swimming muscle mass, (iv) escape performance (escape swimming speed), and (v) a set of physiological traits. We will focus on two fitness-related

physiological traits: investment in immune function (the activity of phenoloxidase, an important enzyme of the immune response in insects, Gonzalez-Santoyo and Cordoba-Aguilar 2012), and oxidative damage to lipids and proteins. As study animals, we use damselfly larvae, important intermediate predators in aquatic food webs, being predators of small invertebrates (e.g. mosquito larvae) and prey for larger organisms (e.g. fish and dragonfly larvae) (Corbet 1999). The response to predation risk of damselfly larvae has been well characterized in terms of life history, behaviour and physiology (e.g. Stoks et al. 2005a, b, 2006; Campero et al. 2007; Slos and Stoks 2008; Janssens and Stoks 2013b). Recently, we also found effects of exposure to non-pathogenic bacteria on behaviour and immune function (Janssens and Stoks 2014), making these ideal organisms to test for potential reinforcing effects of both natural stressors in aquatic ecosystems.

Materials and methods

Experimental setup

We collected penultimate instar larvae of *Coenagrium puella* in a fishless pond with large larval dragonflies as main predators. Although we have no information about the presence of *E. coli* (or other microbiota) at the collecting site, given this is a widespread aquatic bacterium (Espinoza-Urgel and Kolter 1998), it is very likely to be present in all water bodies where the studied damselfly species occurs. The larvae were acclimated in the laboratory under standard conditions of light (14:10 L:D), temperature (22°C) and food (average daily dose of *Artemia* nauplii = 604, SE = 36, N = 10 daily doses, each dose collected on a different day) for at least 15 days. Previous experiments on damselflies showed consistent and comparable effects of predation risk on growth rate (e.g. McPeek et al. 2001) and physiology (e.g. Slos and Stoks 2008) after shorter acclimation periods, and this irrespective of prior experienced predation risk in the field. Moreover, we have shown similar

responses in damselfly larvae that were raised in isolation from the egg stage in the laboratory: for example a decreased growth rate when exposed to bacteria (Janssens and Stoks 2013c) and predation risk (Janssens and Stoks 2013a, b); activation of the immune response when exposed to bacteria (Janssens and Stoks 2013c, 2014); and decreased antioxidant defence and increased oxidative damage when exposed to predation risk (Janssens and Stoks 2013a). This indicates that there is no strong effect of the pre-exposure conditions on the response to predation risk and bacteria in damselfly larvae and that irrespective of any field experience prior to testing, the expected response patterns toward predators and bacteria can be observed.

To test for effects of predation risk, bacterial exposure and their potential interactions, we set up a full factorial design with all four combinations of two predation risk treatments (predator cues absent and present) and two bacterium treatments (*E. coli* absent and present). The day after the larvae moulted into the final instar, we exposed them for seven days and daily refreshed the medium to prevent accumulation of the bacterium. We obtained 30 replicates for both treatments without bacterial exposure; and 40 replicates for both treatments with bacterial exposure (total of 140 larvae). The latter number was higher because 10 larvae of the treatments with bacterial exposure were used to quantify bacterial load. To obtain these replicate numbers at the end of the 7-day exposure period (when end points could be quantified) more larvae had to be started given some mortality occurred during the exposure period. For exact numbers of larvae started per treatment combination see the results section.

One day after larvae moulted into the final instar, they were randomly allocated to one of the four treatment combinations for seven days. There were no differences in initial mass between the different conditions. During the exposure period, larvae were placed individually in glass vials (100 ml) filled with 50 ml of the medium. Glass vials were placed in groups of four in larger containers (750 ml). Each container was allocated to one predation risk

152 treatment. To avoid any bias due to a specific predator or container, we randomly re-
153 distributed vials among containers of the same predation risk treatment on a daily basis.
154 Throughout the exposure period, larvae were daily fed ad libitum with *Artemia* nauplii.
155
156 Exposure to predation risk
157 Predation risk was manipulated using a combination of visual and chemical predator cues,
158 reflecting the cocktail of predator cues that damselfly larvae encounter in nature. To ensure
159 visual predation cues, a large *Anax* dragonfly larva, important predators of damselfly larvae
160 (Stoks et al. 2005a), was placed in the containers of the treatment with predator cues.
161 Additionally, larvae could see the conspecific larvae in the other vials in the container
162 (damselfly larvae are cannibalistic; De Block and Stoks 2004). To avoid visual predator cues
163 in the treatment without predator cues, the walls of these vials were made non-transparent
164 using tape. For the chemical predator cues, we homogenized one *C. puella* larva in 20 ml of
165 water from an aquarium filled with 300 ml aged tap water (i.e. tap water that was aerated and
166 filtered with a carbon filter for at least 24 h) in which a large *Anax* dragonfly larva had eaten a
167 larva of *C. puella*. We daily added 1 ml of this predator medium to each vial of the treatment
168 combinations with predator cues. To the vials of the treatment combinations without predator
169 cues we daily added 1 ml of aged tap water.
170
171 Bacterial exposure
172 As bacterium we used *Escherichia coli*, a widespread bacterium in aquatic invertebrates
173 (McEwen and Leff 2001). More specifically we used the non-pathogenic strain ATCC 11775
174 (Guerrero-Beltran and Barbosa-Canovas 2005). This strain was genetically modified by
175 insertion of a plasmid coding for *dsRed* fluorochrome and kanamycin resistance, allowing it
176 to grow on a culture medium containing kanamycin. This enables us to directly quantify the

177 bacterial load inside the damselfly larvae resulting from the uptake of experimentally
178 provided *E. coli* bacteria (pink colonies) without confounding with the background bacterial
179 load.

180 Throughout the experiment, we daily prepared an *E. coli* solution with a concentration
181 of 1 x 10^9 CFU / 100 ml in mili-Q water using a spectrophotometer. We added 1 ml of this
182 solution to the vials of the bacterial condition resulting in a concentration of 2 x 10^7 CFU 100
183 ml^{-1}. To the control condition we added 1 ml of mili-Q water. We chose this concentration as
184 we have previously shown reduced growth rates in the study species at this concentration
185 (Janssens and Stoks 2013c) and because this is a concentration of non-pathogenic bacteria
186 regularly encountered in water bodies. This concentration of *E. coli* is in the upper range of
187 concentrations for this particular bacterial species reported in field studies (e.g. Kulkoyluoglu
188 et al. 2007), including Flemish surface waters (Aquafin 2012). Total bacterial concentrations
189 reported in natural unpolluted aquatic systems, however, regularly exceed 10^9 CFU 100 ml^{-1}
190 (Kirschner and Velimirov 1997; Palijan 2012). As the large majority of natural aquatic
191 bacterial communities consist of non-pathogenic bacteria, it is likely that damselfly larvae will
192 be frequently exposed to the concentration of non-pathogenic bacteria we used.
193
194 Response variables
195 We daily checked survival during the 7-day exposure period. To quantify growth rate, we
196 weighed each larva to the nearest 0.01 mg at the start and at the end of this period. Growth
197 rate was calculated as (final mass – initial mass) / 7 days.

198 On day four of the 7-day exposure period, we quantified food intake by counting the
199 number of *Artemia* nauplii that each larva consumed during two hours. Per day that we
200 measured foraging activity, we stored the number of *Artemia* nauplii of two initial food
201 aliquots in 70% ethanol to afterwards estimate the average amount of nauplii given to each

larva that day. At the end of each 2h-foraging period, we collected the remaining *Artemia* nauplii per vial and stored them in 70% ethanol. Afterwards, fixated nauplii were counted at magnification 10× using a stereomicroscope. The number of nauplii eaten by a larva on day four was calculated as the difference between the mean initial number given at that feeding day and the number of remaining nauplii in the vial of that larva and expressed as the number of *Artemia* nauplii eaten per hour.

At the end of the 7-day exposure period we measured the escape swimming speed of each larva. Damselfly larvae use swimming to escape predation by dragonfly larvae (Stoks and McPeek 2006; Strobbe et al. 2009). Therefore, we transferred a single larva to a container (20 cm x 12 cm x 8 cm), filled with 1 l of aged tap water. After 10 minutes of acclimation, we scored swimming performance using the methodology of McPeek et al. (1996) and Stoks and McPeek (2006). The larva was stimulated to swim by tapping it on the dorsal surface of the thorax with a plastic pipette. Three swimming bouts per larva were filmed using a high speed camera (Basler pi A 640, 200 Hz) connected to a computer using Streampix software. From these recordings we quantified per swimming bout the swimming speed using Image Pro Plus v5. Swimming speed (cm/s) was calculated as the distance the larvae covered during the first 100 frames of one swimming bout divided by the duration (0.5 s). We chose to digitize the first 0.5 s to have enough frames to accurately calculate swimming speed while this initial 0.5 s period is likely to be the most critical period for damselfly larvae to escape attacks from sit-and-wait predators such as dragonfly larvae that do not chase their prey after the initial attack (Dayton et al. 2005). Per larva, we averaged the speed of the three swimming bouts for later analysis.

Afterwards, ten larvae of the two treatments with the bacterium were used to quantify internal load of the administered non-pathogenic *E. coli* strain ('bacterial load'). These larvae were washed in ethanol, homogenized and the supernatant was used for inoculation on a

culture medium (LB-agar) containing kanamycin. Only the administered kanamycin-resistant *E. coli* strain is able to grow on this culture medium. Each sample was inoculated in duplicate on separate agar plates at each of three serially diluted assay concentrations: (1) 100 µl of the supernatant, (2) 100 µl of a ten times diluted supernatant, (3) 100 µl of a hundred times diluted supernatant. After inoculation, the agar plates were incubated at 28°C for 48 hours. Afterwards, the number of colonies was counted at the assay concentration that had between 10 and 200 colonies. We quantified bacterial load as the mean number of CFU of both replicates and expressed it as CFU per larva.

The remaining 30 larvae per treatment combination were stored at -80°C for quantification of the biochemical/physiological variables. We used 20 larvae per treatment combination (total of 80 larvae) to quantify the effects of predation risk and bacterial exposure on the phenoloxidase activity, a key component of an insect's immune system (Gonzalez-Santoyo and Cordoba-Aguilar 2012). The larvae were 15 times diluted, homogenized in phosphate buffer (PBS, 50mM, pH 7.4) using a pestle and centrifuged (16,100 g, 4 °C). The PO activity was quantified using a modified version of the protocol of Stoks et al. (2006). A 96 well microtiter plate was filled with 50 µl of the supernatant and 25 µl phosphate buffer PBS. Then, we added 5 µl of chymotrypsin (5 mg/ml mili-Q water) and incubated the mixture for 5 minutes at room temperature. This way all of the present pro-enzyme proPO was converted into PO. In a final step, we added 120 µl L-DOPA (10 mM in PBS). We measured the absorbance at 490 nm and at 30 °C every 20 seconds during 30 minutes. PO activity was quantified as the slope of the linear part (500-1500 s) of the reaction curve. Measurements were run in duplicate and the mean of both readings per larva was taken. PO activity was expressed in units with 1 unit representing 1 mmol dopachrome formed per minute per mg protein.

In order to quantify the effects of predation risk and bacterial exposure on protein and lipid content and oxidative damage to proteins an lipids, we used the remaining ten larvae per treatment combination (total of 40 larvae). The abdomen was 15 times diluted, homogenized in phosphate buffer (PBS, 50mM, pH 7.4) using a pestle and centrifuged (16,100 g, 4 °C). The resulting supernatant was used for the physiological analyses.

As swimming muscles of damselfly larvae make up the largest component of the abdomen, we estimated the amount of swimming muscles by quantifying the protein content of the abdomen (cf. Iserbyt et al. 2012). For this, we used the protocol of Bradford (1976). We mixed 1 µl supernatant, 160 µl mili-Q water and 40 µl Bio-Rad Protein Dye in a 96 well plate. After an incubation of 5 minutes at 30°C, we measured absorbance at 595 nm. We calculated protein content based on a standard curve of known concentrations of bovine serum albumin.

To assess the effects of exposure to the bacterium and predation risk in terms of oxidative damage, we quantified oxidative damage to proteins and lipids in ten larvae per treatment combination. To measure oxidative damage to proteins, we quantified one of the most often used biomarkers for this, the level of carbonyls (Monaghan et al. 2009). Carbonyls are introduced into proteins by direct oxidation of amino acids or indirectly by attachment of a carbonyl-containing moiety. The carbonyl content was quantified using the OxiSelect^{TM} Protein Carbonyl ELISA kit STA-310 of Cell Biolabs Inc. In a first step, the supernatant was diluted to obtain a protein concentration of 10 µg/ml. Afterwards, 100 µl of the diluted supernatant was added to a 96 well protein binding plate. After an overnight incubation at 4°C, the wells were washed three times with 250 µl PBS. Next, 100 µl DNPH working solution was added. After an incubation of 45 minutes at room temperature in the dark, the wells were washed five times with 250 µl PBS/ethanol (1:1 v/v) with incubation on an orbital shaker for 5 minutes and two times with 250 µl PBS. From this point the samples were always

incubated on an orbital shaker. Afterwards, 200 µl blocking solution was added and the samples were incubated for 1.5 h at room temperature. After washing the samples three times with 250 µl wash buffer, 100 µl diluted anti-DNP antibody was added and the mixture was incubated at room temperature for 1 hour. Then, the samples were again washed three times with 250 µl wash buffer and 100 µl diluted HRP conjugated secondary antibody was added. After an incubation of 1 hour at room temperature, the samples were washed 5 times with 250 µl wash buffer and 100 µl substrate solution was added. The mixture was incubated for 20 minutes at room temperature The enzymatic reaction was then stopped by adding 100 µl Stop Solution and absorbance was measured at 450 nm. The carbonyl concentrations were calculated based on a standard curve of known concentrations of reduced and oxidized BSA and expressed as nmol carbonyls/mg protein.

We measured oxidative damage to lipids by measuring an often used biomarker of lipid peroxidation, the formation of malondialdehyde (MDA) (Monaghan et al. 2009). Sample preparation was based on the protocol described in Miyamoto et al. (2011). First, 50 µl supernatant and 50 µl TBA 0.4% were mixed (40 mg TBA in 10 ml 0.2 M HCl). This mixture was incubated at 90°C for 60 minutes and cooled on ice. Afterwards, we added 165 µl n-butanol, mixed and centrifuged the mixture for 3 minutes (4°C, 3,300 rpm). Finally, 10 µl of the butanol fraction was injected in an HPLC/UV-Vis system on a C18 column (250 x 4.6 x 5 µm) (protocol by Karatas et al. 2002). The mobile phase was 30 mM KH_2PO_4-methanol (65 + 35, v/v %, pH 4); the flow rate was isocratic, 1 ml/min. Chromatograms were monitored at 535 nm and the retention time of MDA was 3.88 min. A standard curve was established using 1,1,3,3-tetraethoxypropane (TEP, malonaldehyde, bisdiethylacetal). Additionally, fat content was measured following the protocol of Bligh and Dyer (1959). MDA concentrations were expressed in nmol MDA/mg fat.

301 Statistical analyses

302 Given the very few larvae that died in most treatments (see results) it was not possible to
303 analyze the survival data using a logistic regression, instead we performed a series of Fisher
304 exact tests. We used separate AN(C)OVAs to test for the effects of bacterial exposure and
305 predation risk on the other response variables. Interactions were further explored using
306 Duncan posthoc tests. For food intake and internal bacterial load we included the mass of the
307 animals as a covariate. We initially included body mass as a covariate when testing for effects
308 on swimming speed, PO activity and oxidative damage, but since it was not significant (all P
309 > 0.12), it was removed from the final model.

310

311 **Results**

312 Life history and food intake

313 No larvae died in the control (0 out of 30) and very few in the single stressor treatments (only
314 predation risk: 2 out of 32, only exposure to the bacterium: 2 out of 42). Yet, mortality was
315 higher in the treatment where larvae were exposed to both stressors simultaneously and where
316 9 out of 49 larvae died compared to 4 out of 104 in the other three treatment combinations
317 (Fisher exact test, $P = 0.0046$).

318 Overall, exposure to the bacterium ($F_{1, 136} = 43.41$, $P < 0.001$) and to predation risk
319 ($F_{1, 136} = 52.29$, $P < 0.001$) reduced growth rate (Fig. 1a). The growth reduction was the
320 largest when both stressors were combined as indicated by the significant bacterium ×
321 predation risk interaction ($F_{1, 136} = 9.83$, $P = 0.021$).

322 The number of *E. coli* RFP colonies was approximately two times higher when larvae
323 were exposed to predation risk (CFU without predation risk: 948 ± 50, with predation risk:
324 1848 ± 52) ($F_{1, 17} = 6.45$, $P = 0.020$).

Under predation risk larvae reduced food intake ($F_{1, 135} = 4.63$, $P = 0.033$), but only in the absence of the bacterium (bacterium × predation: $F_{1, 135} = 5.37$, $P = 0.022$) (Fig. 1b). From the point of view of the bacterium, *E. coli* had no effect on the foraging activity in the absence of predation risk ($P = 0.21$) and a positive effect when predation risk was present ($P = 0.037$). Larger larvae ate more ($F_{1, 135} = 8.30$, $P = 0.0046$; slope ± 1 SE = 8.83 ± 3.07).

Muscle mass and escape swimming speed

Exposure to the bacterium caused a lower abdominal muscle mass ($F_{1, 36} = 50.97$, $P < 0.001$) (without predation risk $P = 0.0021$, with predation risk $P < 0.001$), and this was more pronounced in the presence of predation risk as indicated by the bacterium × predation risk interaction ($F_{1, 36} = 5.15$, $P = 0.029$, Fig. 2a). From the point of view of predation risk, predation risk decreased the abdominal muscle mass only in the presence of the bacterium (without bacterium $P = 0.48$, with bacterium $P < 0.001$).

There was a significant interaction between bacterial exposure and predation risk for swimming speed ($F_{1, 136} = 12.01$, $P < 0.001$; Fig. 2b). Although the bacterium caused a lower swimming speed in both predation risk treatments ($F_{1, 136} = 46.32$, $P < 0.001$) (without predation risk $P = 0.044$; with predation risk $P < 0.001$), this reduction was stronger when predation risk was also present. Predation risk only resulted in slower swimming when the bacterium was also present (without bacterium $P = 0.64$; with bacterium $P < 0.001$) (Fig. 2b).

Physiological variables

Predation risk and exposure to the bacterium interacted with each other for the PO activity ($F_{1, 76} = 5.11$, $P = 0.027$; Fig. 3a). In larvae exposed to the bacterium PO activity increased in the absence of predation risk ($P = 0.011$), yet it decreased when predation risk was present ($P = 0.046$).

There was a significant interaction between bacterial exposure and predation risk for the carbonyl levels ($F_{1, 36} = 4.24$, $P = 0.047$; Fig. 3b). Both exposure to the bacterium ($F_{1, 36} = 71.91$, $P < 0.001$) and to predation risk ($F_{1, 36} = 65.96$, $P < 0.001$) resulted in increased carbonyl levels, but this increase was the largest when both stressors were combined.

Exposure to the bacterium ($F_{1, 36} = 29.43$, $P < 0.001$) and to predation risk ($F_{1, 36} = 30.47$, $P < 0.001$) resulted in higher MDA levels, in an additive way (bacterium x predation: $F_{1, 36} = 1.12$, $P = 0.30$) (Fig. 3c).

Discussion

Life history variables and immune function

We found synergistic effects for all three life history variables (mortality, growth rate and bacterial load) when larvae were simultaneously exposed to the bacterium and to predation risk. While exposure to predation risk or to the bacterium did not cause mortality, significant mortality occurred when both non-lethal stressors were combined. A similar synergistic increase of mortality has been shown when predation risk was combined with a virus (e.g. Kerby et al. 2011). Importantly, as we used a non-pathogenic *E. coli* strain the increased mortality cannot be explained by pathogenicity and can be directly linked to the response of the host itself. This increased mortality could be caused by a reduction of the immune defence when both stressors were combined (as indicated by the reduction in PO activity), resulting in a too high accumulation of the bacterium. Non-pathogenic bacteria (Freitak et al. 2007, 2009) have indeed been shown to reduce fitness components. Additionally, the highest levels of oxidative damage when both stressors were combined may have become lethal by impairing critical functions such as signal transduction and the functioning of receptors, antibodies and transport proteins and enzymes (Halliwell and Gutteridge 2007).

As often demonstrated (reviewed in Benard et al. 2004), exposure to predation risk resulted in a lower growth rate. This can be explained by the observed lower food intake under predation risk. Yet, also a lower conversion of food into biomass likely played a role (e.g. McPeek et al. 2001; Stoks et al. 2005b; Trussell et al. 2006). Under predation risk, prey organisms such as damselfly larvae, are known to shunt more energy toward the fight-or-flight response to mobilize energy for escape burst swimming (Strobbe et al. 2010) and toward the upregulation of costly defence mechanisms (for example the levels of Hsp70; Pauwels et al. 2005; Slos and Stoks 2008). Exposure to the non-pathogenic *E. coli* strain also caused lower growth rates, thereby adding to the few other studies documenting sublethal fitness effects of non-pathogenic bacteria (Freitak et al. 2007, 2009; Janssens and Stoks 2013c). As larvae experimentally exposed to *E. coli* did not decrease their food intake, the bacterium-induced growth reduction is entirely physiologically mediated, probably through investment in similar defence mechanisms as under predation risk (e.g. Reilly et al. 2007; Freitak et al. 2007, 2009; Janssens and Stoks 2013c). Additionally, competition between the natural symbiotic midgut microbiota and the bacteria admitted to the damselfly larvae (Smith and Holt 1996) and changes of the abiotic environment by the bacterium (Ansa et al. 2011) may have contributed to the observed growth reduction. This growth reduction was the strongest when both stressors were present, probably due to energetic constraints.

An important finding of our study is that predation risk increased the bacterial load in the damselfly larvae. The only other study looking at the effect of predation risk on bacterial load found no effect (Haislip et al. 2012). A possible explanation for our observation is that due to the shunting of energy to the fight-or-flight response in response to predation risk (Slos and Stoks 2008), the animals invested less in immune function, resulting in higher levels of the bacterium. This hypothesis is confirmed by the observation that larvae jointly exposed to the bacterium and predation risk had a lower PO activity. A decrease in immune function in

399 the presence of predation risk has been observed before (e.g. Rigby and Jokela 2000; Stoks et
400 al. 2006; Pauwels et al. 2010).
401
402 Behavioural antipredator responses
403 The combined exposure to the bacterium and predation risk considerably impaired the two
404 key antipredator mechanisms of the damselfly larvae: they no longer reduced their food intake
405 under predation risk and showed a synergistic reduction in escape swimming speed. This in
406 contrast with larvae exposed to predation risk in the absence of the bacterium which lowered
407 their foraging activity. This is an important adaptive strategy to avoid detection by the
408 visually orientated dragonfly predators (Werner and Anholt 1993; for damselfly larvae see
409 e.g. Stoks et al. 2003; Janssens and Stoks 2012). It is likely that the larvae that were exposed
410 to both stressors invested a lot of energy in costly defence mechanisms (see above). In order
411 to meet those energetic demands, they needed to forage more actively, even when this came at
412 the cost of increased detection by the predator.
413 Once detected, damselfly larvae rely on escape swimming to avoid being killed by an
414 attacking predator. The escape speed was strongly reduced in the presence of the bacterium
415 and especially so when both stressors were combined. This reduction of escape speed can be
416 explained by three mechanisms: (i) a higher investment in costly physiological defence
417 mechanisms (e.g. upregulation of Hsp70 in presence of predation risk ([e.g. Pauwels et al.
418 2005; Slos and Stoks 2008] and bacteria [e.g. Reilly et al. 2007; Janssens and Stoks 2013c]),
419 (ii) a lower swimming muscle mass (this study) and (iii) the accumulation of oxidative
420 damage (this study, see below).
421
422 Oxidative damage

Both predation risk and the bacterium caused oxidative damage to lipids and to proteins as indicated by the higher levels of MDA and carbonyl, respectively. We here for the first time studied the combined effect of these widespread natural stressors in aquatic ecosystems and could thereby demonstrate for the first time a synergistic increase in oxidative damage (as measured by carbonyl levels) in the combined presence of both stressors.

Although several studies have shown decreased antioxidant defence when prey are exposed to predation risk (e.g. Slos and Stoks 2008; Travers et al. 2010; Trekels et al. 2012), studies showing an associated increase in oxidative damage are rare (Guerra et al. 2013; Janssens and Stoks 2013a). We hypothesize such predator-induced increases of oxidative damage to be widespread in prey given the generality of the combination of the two assumed underlying mechanisms. First, in response to predation risk, prey organisms typically increase their metabolic rate (e.g. Beckerman et al. 2007; Slos and Stoks 2008). Second, prey organisms have been shown to reduce antioxidant defence under predation risk (e.g. Slos and Stoks 2008; Travers et al. 2010; Trekels et al. 2012; Guerra et al. 2013; Janssens and Stoks 2013a).

We extended previous observations that bacteria can cause oxidative damage (e.g. Baik et al. 1996; Castex et al. 2009; Wang et al. 2012), including a study showing higher MDA levels in animals exposed to a pathogenic *E.coli* strain (Liu et al. 2009), by showing this may also occur in response to living non-pathogenic bacteria. The higher levels of MDA and carbonyl in larvae exposed to bacteria can be explained by the increased ROS concentrations in the host in the presence of bacteria (for example, due to an activation of the immune system, Steinberg 2012) or by ROS production by the bacteria themselves (Shaked and Rose 2013).

Conclusions and implications

448 Evidence is accumulating that natural stressors may synergistically interact thereby creating
449 ecological surprises (Darling and Côté 2008). Most studies investigating the effects of
450 multiple stressors only considered life history variables, while physiology and performance
451 are often ignored (Darling and Côté 2008). We here demonstrated for the first time
452 reinforcing effects between stress imposed by non-pathogenic bacteria and by predators, two
453 widespread stressors in aquatic systems (Kerfoot and Sih 1987; Leff and Lemke 1998), on
454 different trait types likely to affect fitness in prey organisms. While the most striking effect
455 was that non-pathogenic bacteria became lethal in the presence of predation risk, we also
456 documented synergistic effects on other life history traits (growth rate and bacterial load).
457 Moreover, the reinforcing negative effect on escape speed is expected to translate into an
458 increased vulnerability to predation. The mechanisms identified here that might explain the
459 observed synergistic interactions between non-pathogenic bacteria and predation risk,
460 including a reduced food intake, an impaired investment in immune function and increased
461 oxidative damage, are very general and are likely to also occur when predation risk is
462 combined with other micro-organisms, including mixtures of micro-organisms (e.g. also
463 exposure to the gram-positive bacterium *Brevibacterium* resulted in increased ROS
464 production and oxidative damage [Zhang et al., 2013]). We therefore hypothesize that the
465 here observed synergistic effects on life history and escape performance may be widespread
466 and that the ignored ecological role of non-pathogenic bacteria in reinforcing the effects of
467 predation risk deserves more attention. This is especially true as recent studies indicated that
468 the effects of predation risk may strongly shape prey population dynamics (McCauley et al
469 2011), community structure (Peacor et al. 2012) and ecosystem functions (Hawlena et al.
470 2010).
471
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647

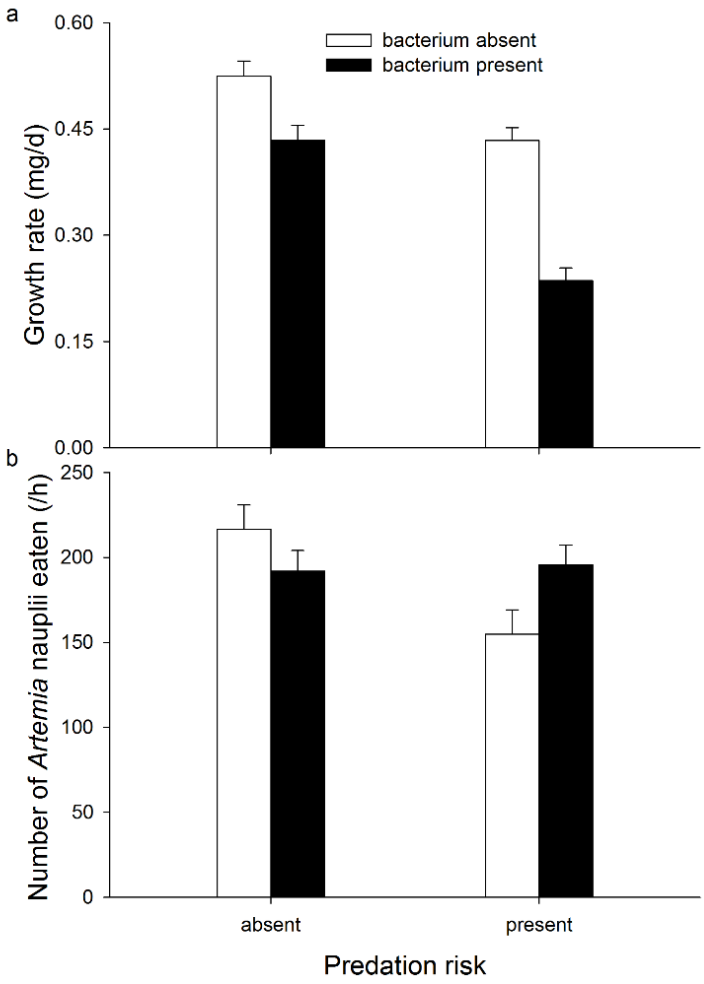
Figure Legends

Fig. 1 Effects of exposure to predation risk and *E. coli* on (a) growth rate (expressed as mass gain per day) (n = 40) and (b) food intake (n = 40) in *Coenagrion puella* damselfly larvae. Given are least-squares means + 1 SE

Fig. 2 Effects of exposure to predation risk and *E. coli* on (a) abdominal muscle mass (n = 10) and (b) escape swimming speed (n = 40) in *Coenagrion puella* damselfly larvae. Given are least-squares means + 1 SE

Fig. 3 Effects of exposure to predation risk and *E. coli* on (a) PO activity (n = 20), (b) oxidative damage to proteins (carbonyl levels) (n = 10) and (c) oxidative damage to lipids (MDA levels) (n = 10) in *Coenagrion puella* damselfly larvae. PO activity is expressed in units with 1 unit representing 1 mmol dopachrome formed per minute per mg protein. Given are least-squares means + 1 SE

669 **Fig. 1**



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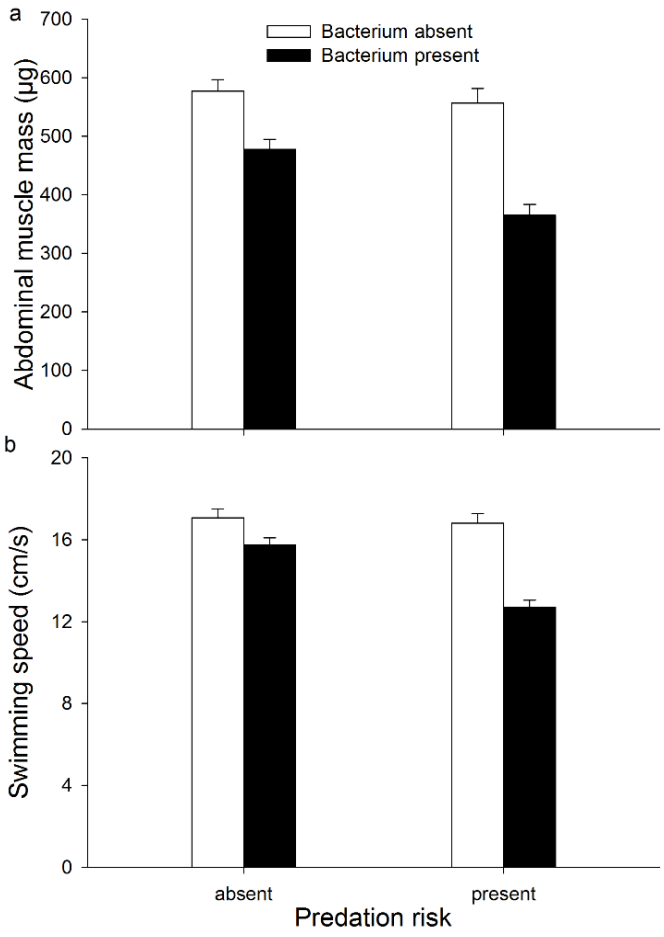
672 **Fig. 2**



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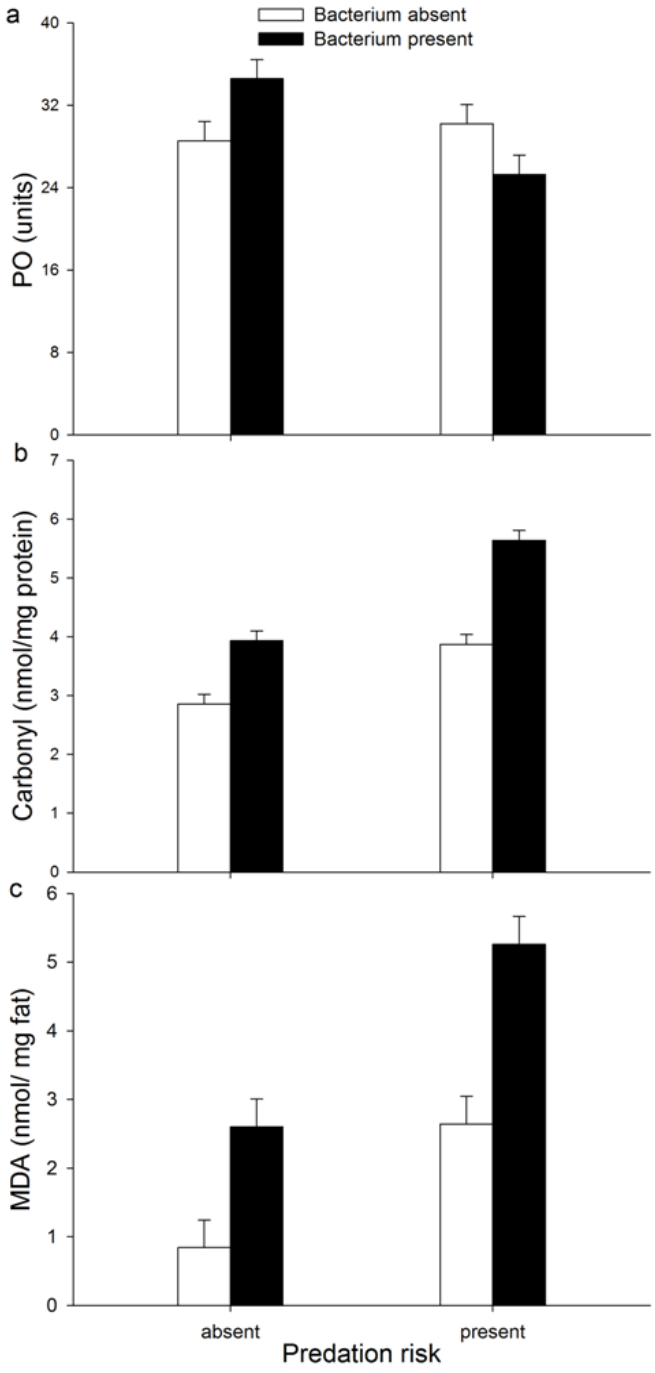
675 **Fig. 3**



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